Organophosphorous Neuropathy

I. A Teased-Fiber Study of the Spatio-Temporal Spread of Axonal Degeneration

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The spatio-temporal spread of axonal degeneration in organophosphorous neuropathy has been studied by means of the teased-fiber technique. Young adult cats were given a single intraperitoneal injection of di-isopropylfluorophosphate (DFP) and were killed 14, 18, 20, 21, and 28 days later by intracardiac perfusion with aldehydes. The cats developed clinical signs of delayed neurotoxicity 16 to 18 days after DFP injection. A histologic survey of the central and peripheral nervous systems revealed that the topographic distribution of axonal degeneration was characteristic of a dying-back neuropathy. In teased-fiber preparations from the left recurrent laryngeal nerve, we found that the axonal degeneration was initially focal and nonterminal but that the axonal degeneration subsequently spread in a somatofugal direction to involve the entire distal axon. Nerve fiber varicosities and paranodal demyelination preceded the axonal degeneration. It is concluded that neurotoxic organophosphates induce a focal, distal but not terminal, axonal degeneration. This "chemical transection" of the axon then precipitates wallerian degeneration of the more distal axon. Thus, the traditional hypothesis that dying-back neuropathies evolve from a retrograde axonal degeneration is not valid for organophosphorous neuropathy. (Am J Pathol 94:241–252, 1979)

CERTAIN ORGANOPHOSPHATES are well known for producing a "dying-back" neuropathy in humans and animals.[1] Although the pathogenesis of organophosphate-induced axonal degeneration remains unknown, light and electron microscopic studies have established that the axonal degeneration primarily affects the distal ends of the longest and largest nerve fibers.[1,2] None of these studies, however, has clearly established the spatio-temporal evolution of the organophosphate-induced axonal degeneration. Yet a knowledge of the evolution of the axonal degeneration is important for several reasons: First, establishment of the initial locus of axonal degeneration may give insight into the mechanism of action of the organophosphates. Second, although the spatio-temporal pattern of wallerian degeneration has been extensively studied,[3,4] the spatio-temporal pattern of non-wallerian forms of axonal degeneration has received little attention. Third, recent findings from studies of the giant

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axonal neuropathies have raised the question whether the axonal degeneration in dying-back neuropathies actually begins at the axon's terminus and proceeds somatopetally.[5]

The teased-fiber technique is ideal for studying the spatio-temporal evolution of axonal degeneration because this technique permits one to assess individual nerve fibers, maintained in their proper proximo-distal orientation, over reasonably long distances (1 to 1.5 cm). We employ the teased-fiber technique in this communication to define the spatio-temporal evolution of axonal degeneration induced by the organophosphate di-isopropylfluorophosphate (DFP). A companion paper elucidates the fine-structural evolution of the axonal degeneration.[6]

Materials and Methods

Animal Model

Young adult, male and female, random-bred cats, weighing 2.6 to 3.1 kg and specified pathogen-free, were housed in wire-bottomed cages and permitted cat chow and water *ad libitum*. Seven cats were used in this study. The cats were given a single intraperitoneal injection of di-isopropylfluorophosphate (DFP) (Aldrich Chemical Company, Ltd.), 40 mg/kg. The DFP was dissolved in sterile water (15 mg/ml). Because DFP irreversibly binds acetylcholinesterase, the animals had to be "protected" from fatal acetylcholine poisoning with eserine (1.7 mg/kg) and atropine (0.6 mg/kg),[7] which were injected intraperitoneally as a mixed solution.

Clinical Course

Within a few minutes of the eserine/atropine injection, the cats displayed clinical signs of acetylcholine poisoning: pupillary dilatation and generalized muscular fasciculations. The subsequent DFP injection did not exacerbate these signs of acetylcholine poisoning. By 24 hours after administration of DFP, the cats appeared normal except for minimal hind-limb weakness, which resolved over the next 24 hours. Hind-limb ataxia, the first clinical sign of delayed organophosphorous neurotoxicity, appeared 16 to 18 days after DFP injection and increased to moderate severity over the next 3 to 5 days. After the onset of ataxia, the cats also developed hind-limb hypotonia, hind-limb weakness, and minimally increased deep-tendon reflexes. These signs of neural dysfunction stabilized at 21 to 23 days after DFP injection.

Preparation of Tissues

The recurrent laryngeal nerve was chosen for teased-fiber study because it contains a stable, well-defined, reasonably small population of large myelinated fibers (approximately 350 fibers) throughout its entire length. The recurrent laryngeal nerve is composed of two distinct populations of myelinated nerve fibers: a population of small myelinated fibers (1 to 3 μ in diameter) that innervate the trachea and esophagus and a population of large myelinated fibers (4 to 15 μ in diameter) that travel the entire length of the recurrent laryngeal nerve without significant branching to innervate the larynx.[8,9] Thus, any level of recurrent laryngeal nerve includes the same population of large myelinated nerve fibers as any other level.

For our teased-fiber studies, 5 cats were anesthetized with pentobarbital and perfused through the ascending aorta with 4% glutaraldehyde (in 0.1 M sodium cacodylate, pH 7.4)

at 14, 18, 20, 21, and 28 days after DFP. The entire left recurrent laryngeal nerve (LRLN) was isolated and divided into 1.5-cm segments which were osmicated (1% osmium tetroxide in 0.1 M sodium cacodylate), dehydrated in graded alcohols, and cleared in cedarwood oil. The proximo-distal orientation of each segment was preserved throughout the processing. Individual nerve fibers were isolated, mounted on glass slides, and coverslipped with permanent mounting media. Portions of the LRLN were also embedded in Araldite, sectioned at 0.5 μ, stained with either toluidine blue or ammoniacal silver nitrate (myelin impregnation), and examined by light microscopy.

For a general survey of the pathologic changes in organophosphorous neuropathy, 2 cats were perfused with 10% formol–saline 21 days after DFP injection. Portions of the cerebrum, cerebellar vermis, medulla, spinal cord, lumbar dorsal root ganglia, sciatic nerve, posterior tibial nerve, and plantar nerve were embedded in paraffin, sectioned at 10 μ, and stained with either hematoxylin and eosin, the Glees–Marsland method for axons, or the Klüver-Barrera method for myelin.

Teased-Fiber Study and Quantitation

From each of the 5 glutaraldehyde-fixed animals, single nerve fibers up to 1.5 cm in length were teased from all levels of the LRLN and examined by light microscopy. (These were the same fibers utilized in the quantitative studies described below.) Particular attention was paid to the proximo-distal orientation of individual nerve fibers. The presence of primary myelin ellipoids was considered to be morphologic evidence of axonal degeneration [4]; widening of the nodal gap beyond 10 μ was considered to be morphologic evidence of paranodal demyelination.

For quantitative studies,[10] 50 to 150 single nerve fibers were teased without preselection from each 1.5-cm segment of the LRLN of the 14-, 18-, 21-, and 28-day animals. Each 1.5-cm segment was numbered consecutively; the most proximal 1.5-cm segment was designated "Level 1." The 50 to 150 single nerve fibers from each level of each LRLN were evaluated and assigned to one of four categories: A = normal myelinated nerve fiber; B = fiber with one or more internodes having paranodal demyelination as the only abnormality; C = fiber with one or more internodes having one or more varicosities but with no internodes having primary myelin ellipsoids characteristic of wallerian-type degeneration; paranodal demyelination may or may not be present; D = fiber undergoing wallerian-type degeneration (the axonal degeneration may be localized or diffuse); paranodal demyelination and/or varicosities may or may not be present. Accurate quantitative studies of the LRLN were not possible in the 28-day animal because the large number of degenerating nerve fibers (Büngner bands) could not be adequately separated to give an accurate count of the total number of fibers examined.

Results

General Pathologic Findings

The pathologic changes in the paraffin sections of the central and peripheral nervous systems in the 2 21-day animals had the characteristic distribution of a dying-back neuropathy and were comparable with those reported previously in feline organophosphorous neurotoxicity.[11,12] In brief, degenerating nerve fibers were frequently present in the plantar nerve, much less frequent in the posterior tibial nerve, and rarely present in the sciatic nerve. Within the central nervous system, we found occasional degenerating nerve fibers in the white matter of the cerebellar

vermis. In the cervical spinal cord, nerve fiber degeneration was prominent within the fasciculus gracilis and within the lateral funiculus in the area of the spinocerebellar tracts. In the mid-thoracic spinal cord, there was slight nerve fiber degeneration within the fasciculus gracilis and within the lateral funiculus in the areas of the spinocerebellar tracts and the lateral corticospinal tract. In the lumbar spinal cord, there was prominent nerve fiber degeneration within the lateral funiculus in the area of the lateral corticospinal tract.

There was no evidence of neuronal cell death or central chromatolysis. In particular, the dorsal root ganglion cells, the anterior horn cells, and the neurons of the nucleus ambiguous (nucleus of origin of motor fibers of the recurrent laryngeal nerve) were normal.

Teased-Fiber Studies of LRLN

Axonal degeneration, as identified by the criterion of myelin–ellipsoid formation,[4] was first seen in the LRLN in the 18-day animal. The incidence of degenerating fibers progressively increased in the 18-, 20-, 21-, and 28-day animals (Table 1). The vast majority of degenerating fibers were in the distal half of the LRLN. A striking finding in the teased-fiber preparations was that there were occasional fibers with degenerating (fragmenting) proximal internodes yet completely intact distal internodes. In these fibers having such focal, non-terminal axonal degeneration, the myelin ellipsoids were usually initially localized to the mid-internodal region of one or more internodes (Figure 1). It became evident from our study of a large number of nerve fibers showing progressive stages of axonal degeneration that the axonal degeneration (formation of myelin ellipsoids) spread somatofugally and that the subsequent degeneration of more distal internodes usually commenced with localized, mid-internodal varicosities. When the focal axonal degeneration started to spread somatofugally through the distal axon, the morphologic stages of the axonal degeneration were identical to those described for wallerian degeneration.[4]

Among the fibers showing the later stages of axonal degeneration, there were occasional fibers with intact proximal internodes yet degenerating distal internodes. This definitely established that the degeneration involved only the distal portion of individual axons. Semithin, transverse sections from the proximal and distal ends of the LRLN confirmed that the degeneration involved only the distal axon. Whereas the distal ends of the nerves from the 21- and 28-day animals had marked nerve fiber degeneration (Figure 2A), the proximal ends of the same nerves appeared completely normal (Figure 2B).

Table 1—Distribution of Nerve Fiber Abnormalities at Various Levels of the Left Recurrent Laryngeal Nerve

Days after DFP	Level of nerve*	Type of myelinated nerve fiber (%)†			
		A	B	C	D
14	1	100	0	0	0
	2	99	0	1	0
	3	98	1	1	0
	4	96	2	2	0
	5	98	0	2	0
18	1	96	2	0	2
	2	88	4	4	4
	3	78	5	6	11
	4	65	2	10	23
	5	75	7	3	15
	6	76	1	3	20
	7	63	1	4	32
21	1	91	4	3	2
	2	69	5	7	19
	3	51	4	9	36
	4	51	4	6	39
	5	54	7	1	38

* Level 1 denotes the most proximal 1.5-cm segment of LRLN; Level 5 or 7 denotes the most distal 1.5-cm segment.

† Fifty to one hundred fifty fibers were evaluated at each level and assigned to one of four categories: A, normal fiber; B, fiber with only paranodal demyelination; C, fiber with internodal varicosities (paranodal demyelination may or may not be present, but wallerian-type degeneration is not present); D, fiber undergoing wallerian-type degeneration, which may be localized or diffuse (paranodal demyelination and/or varicosities may or may not be present).

The 14-day animal, which was the earliest animal examined in our series, had no clinical signs of ataxia and had no histologic signs of axonal degeneration in the LRLN. Examination of teased fibers, however, revealed focal nerve fiber varicosities and paranodal demyelination (Figure 3). The nerve fiber varicosities, which gave the involved internodes a "beaded" appearance, were also present in the 18-, 20-, 21-, and 28-day animals, which had definite clinical signs of ataxia and an increasingly large percentage of nerve fibers undergoing axonal degeneration. Quantitative teased-fiber studies (Table 1) in the 14-, 18-, and 21-day animals revealed that the incidence of beaded fibers increased from an average of 1% in the 14-day animal to an average of 5% in the 21-day animal. The varicosities were most numerous in the middle third of the LRLN. Frequently, more than one internode along a fiber was beaded, and it was not unusual for an internode of normal appearance to separate beaded ones. Internodes distal to a beaded internode were often completely unremarkable. The diameters of the varicosities were approximately twice those of

the involved nerve fibers. Transitional stages between varicosities and myelin ellipsoids were occasionally found (Figure 1). It is noted, however, that transitional stages between varicosities and myelin ellipsoids were difficult to interpret because varicosities closely resembled incipient myelin ellipsoids by light microscopy.

Paranodal demyelination, the other morphologic abnormality in the 14-day animal, was also present in the 18-, 20-, 21-, and 28-day animals. Quantitative studies (Table 1) revealed that the incidence of paranodally demyelinated fibers increased from an average of 0.6% in the 14-day animal to an average of 5% in the 21-day animal. Although paranodal demyelination was commonly associated with beaded fibers (Figure 3) or fibers undergoing axonal degeneration, some nerve fibers displayed only paranodal demyelination in one or more internodes. The demyelination did not appear to be random: whereas most fibers had no paranodal demyelination, when an individual fiber had one paranodally demyelinated internode, it frequently had others.

Discussion

The organophosphorous compounds are part of a growing list of neurotoxic chemicals that produce a characteristic pattern of axonal degeneration. These so-called dying-back neuropathies [1] are characterized by an axonal degeneration involving only the distal portions of axons: the proximal portions of the axons and their perikarya remain intact. Also characteristic of the dying-back neuropathies is the selective involvement of the longer and larger axons. This dying-back pattern of axonal degeneration is usually expressed clinically as a distal, symmetric sensorimotor polyneuropathy. It has been generally assumed, although never proved, that this selective degeneration of the distal axon evolves from a retrograde axonal degeneration, which begins at the axon's terminus and then progresses somatopetally along the distal axon ("dying-back hypothesis").[1] Our general histologic survey of the central and peripheral nervous systems in feline organophosphorous neuropathy illustrates the selective distribution of axonal degeneration to the distal ends of long nerves, which is characteristic of a dying-back neuropathy. However, our teased-fiber study of the LRLN demonstrates that, although the degeneration is limited to the distal axon, the degeneration does not begin at the axon's terminus and progress somatopetally. Instead, DFP induces a focal axonal degeneration that is sited in the distal, but not terminal, axon. Subsequent to this initially very localized axonal degeneration, the axonal degeneration rapidly spreads somatofugally to involve the entire distal axon. These findings demonstrate that the hypothesis of retrograde axonal degenera-

tion is invalid for organophosphorous neuropathy and call into question the validity of the hypothesis for other dying-back neuropathies. In this regard, Spencer and Schaumburg, working with a very different dying-back neuropathy induced by long-chain hydrocarbons, have also questioned the validity of the dying-back hypothesis.[5]

The spatio-temporal evolution of nerve fiber degeneration is strikingly similar in both wallerian degeneration [4] and organophosphorous neuropathy, except for the development of varicosities in the latter. We do not believe that this similarity is coincidental. On the contrary, we propose that the initially focal axonal degeneration induced by the neurotoxic organophosphate precipitates wallerian degeneration of the distal axon. Thus, in effect, DFP, by inducing a focal, non-terminal axonal degeneration, produces a "chemical transection" of the nerve fiber.

The presence of paranodal demyelination in organophosphorous neuropathy may be construed as the result of a direct toxic effect of DFP on Schwann cells. However, the observation that the paranodal demyelination was not random implies that the myelin breakdown was a secondary reaction of the Schwann cell to underlying axonal abnormalities rather than a primary reaction of the Schwann cell to DFP.[13]

The internodal varicosities described here have not been reported previously in either organophosphorous neuropathy, other dying-back neuropathies, or wallerian degeneration. That such varicosities were not noted previously in organophosphorous neuropathy presumably reflects a paucity of teased-fiber studies in these earlier reports. It is not surprising that the varicosities have not been recognized in conventional cross and longitudinal sections of peripheral nerve when one considers the low incidence of beaded nerve fibers, the small size and very localized distribution of the varicosities, and the resemblance of varicosities to incipient myelin ellipsoids. These varicosities are not limited to the LRLN in organophosphorous neuropathy, since we have seen them in teased-fiber preparations of the plantar nerve. Our light microscopic observations suggest that these varicosities evolve into focal axonal degeneration. If this is true, then the varicosities may have an important role in the pathogenesis of organophosphate-induced axonal degeneration. Ultrastructural studies of these varicosities constitute the basis of the companion paper.[6]

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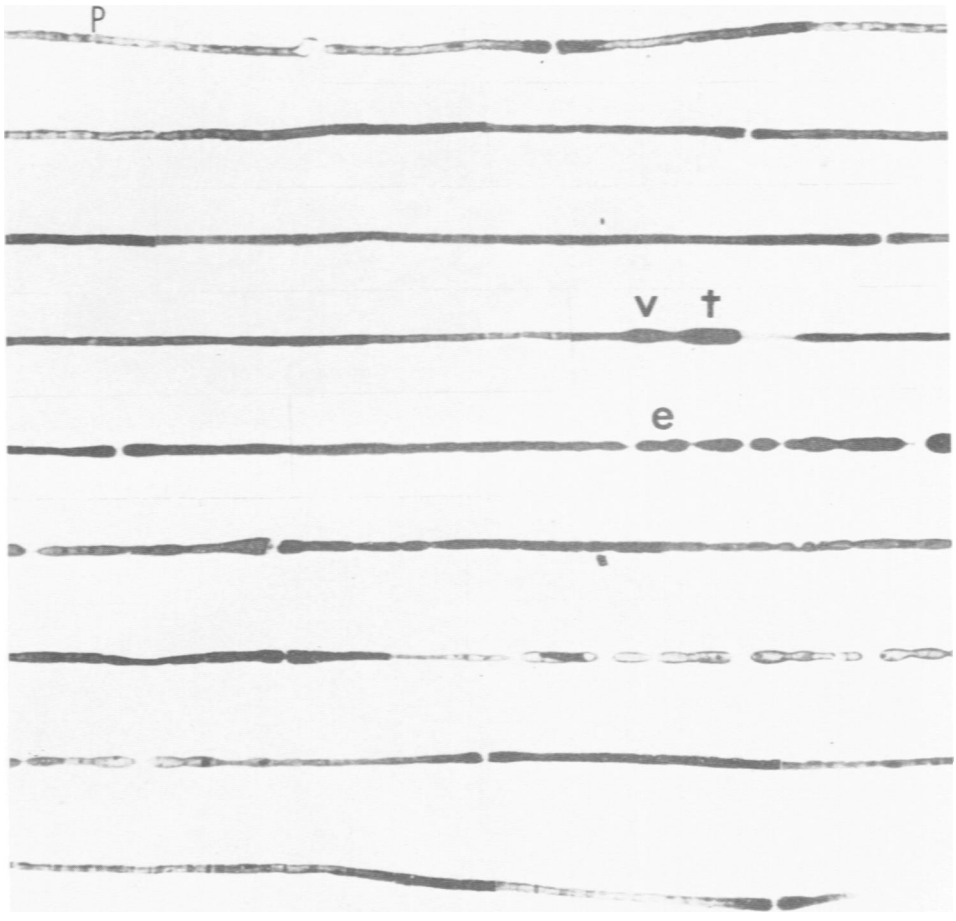

Figure 1—Nine consecutive segments of a single, teased myelinated fiber from the 20-day animal. Axonal degeneration is localized to four contiguous internodes in the fourth through the eighth segments. More proximal internodes (first three segments) and more distal internode (last two segments) are normal. A transitional stage (*t*) between a varicosity (*v*) and a myelin ellipsoid (*e*) is in the most proximal of the degenerating internodes. *P* denotes proximal end of fiber. (Osmium tetroxide, × 112)

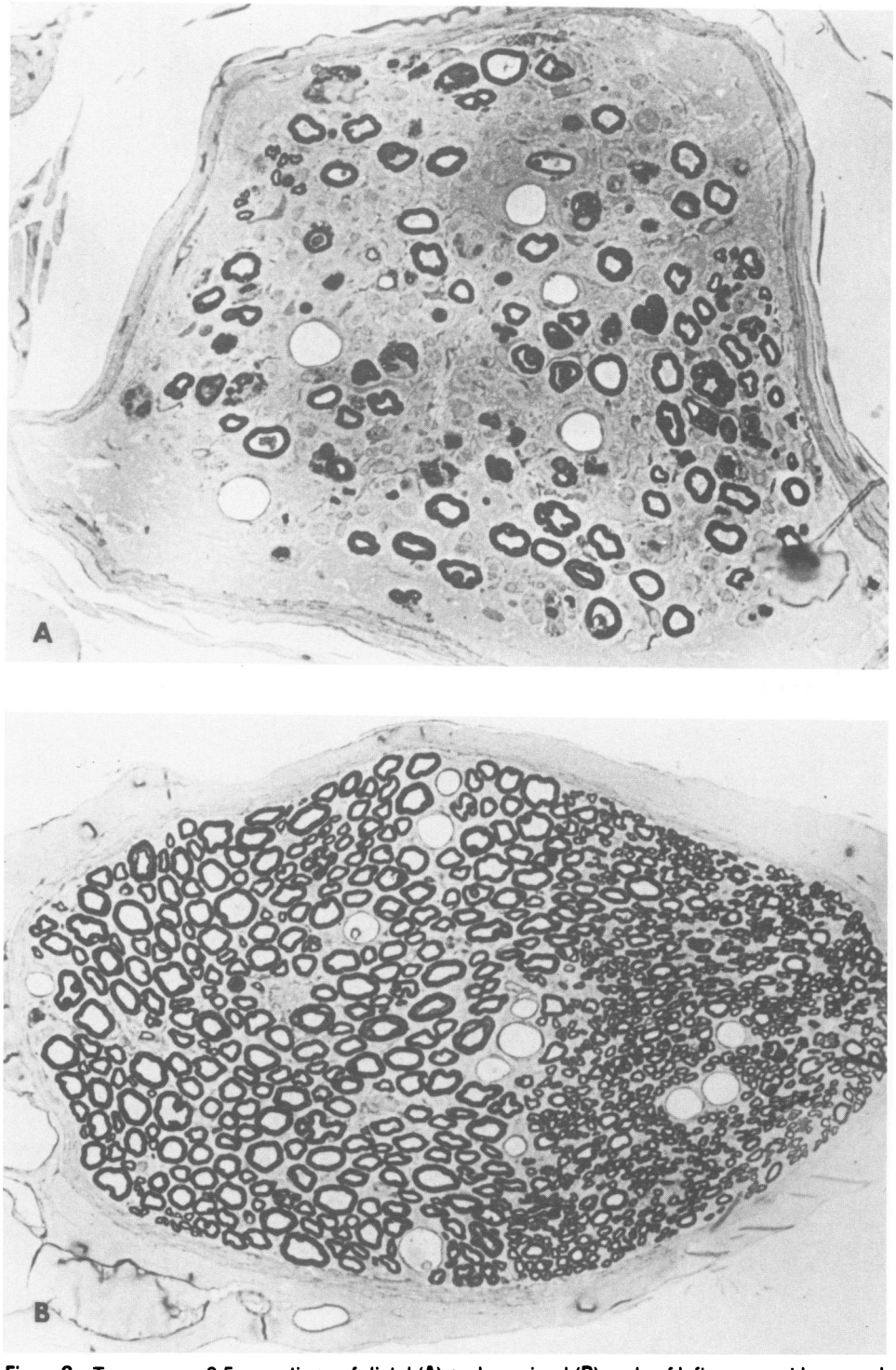

Figure 2—Transverse, 0.5-μ sections of distal (A) and proximal (B) ends of left recurrent laryngeal nerve from 28-day animal. Although the distal end (A) has marked loss and degeneration of fibers, the proximal end (B) appears normal. (Ammoniacal silver nitrate, × 440)

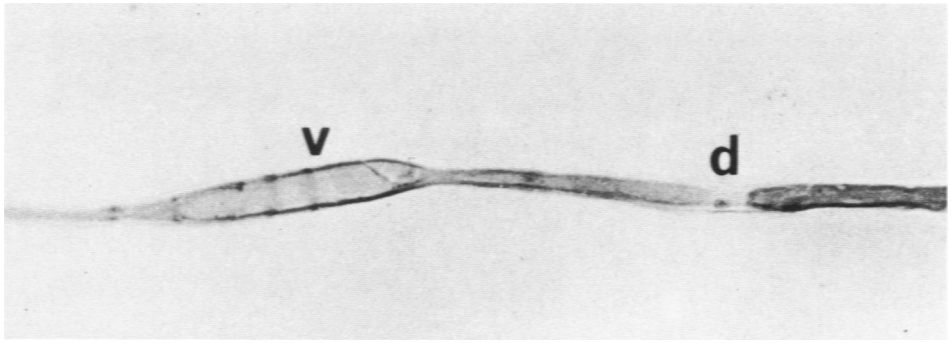

Figure 3—A teased, myelinated nerve fiber showing internodal varicosity (*v*) and paranodal demyelination (*d*) in 14-day animal. (Osmium tetroxide, × 350)

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